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Free radical mediated grafting of chitosan with caffeic and ferulic acids: Structures and antioxidant activity

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ABSTRACT

In this study, two water soluble chitosan derivatives were synthesized by grafting caffeic acid (CA) and ferulic acid (FA) onto chitosan *via* a free radical mediated method. The structural characterization, antioxidant activity *in vitro* and *in vivo* of chitosan derivatives were determined. Results showed that the UV–vis absorption peaks of chitosan derivatives shifted toward longer wavelengths. FT-IR spectroscopy exhibited the typical phenolic characteristics within 1450–1600 cm^{-1}. ^{1}H NMR spectroscopy showed new peaks of phenyl protons at 6.2–7.6 ppm. ^{13}C NMR spectroscopy showed additional peaks between 110 and 150 ppm assigned to the C=C of phenolic groups. These results all confirmed the successful grafting of CA and FA onto chitosan backbones. The chitosan derivatives had decreased thermal stability and crystallinity as compared to chitosan. *In vitro* assays showed that the antioxidant activity decreased in the order of CA-g-chitosan > FA-g-chitosan > chitosan. Moreover, administration of the chitosan derivatives could significantly increase antioxidant enzymes activities and decrease malondialdehyde levels in both serums and livers of D-galactose induced aging mice. Our results indicated the potential of CA-g-chitosan and FA-g-chitosan in the development of novel antioxidant agents.

1. Introduction

There are many kinds of reactive oxygen species (ROS), such as hydrogen peroxide, hydroxyl radical and singlet oxygen. Although ROS at physiological concentration may be required for normal cell functions, excessive amount of ROS can damage cellular components such as lipids, protein and DNA [1]. The free radical theory of aging suggests that the damage produced by the interactions of ROS with cellular macromolecules results in cellular senescence and aging [2]. Most organisms possess antioxidant defense and repair systems to protect them against oxidative damages. However, these systems are insufficient to prevent damages entirely. Therefore, it is essential to develop effective antioxidants to protect human body from free radicals. Recently, several natural polysaccharides and their derivatives have been demonstrated to possess potent antioxidant activities and potential applications as antioxidants [3–5].

Chitosan, a deacetylated derivative of chitin, is the second most abundant polysaccharide found in nature after cellulose. Due to its biocompatible, biodegradable, non-toxic and non-antigenic properties, chitosan has wide applications in many fields including biomedicine, waste water treatment, cosmetics and food industries [6]. However, chitosan is only soluble in some dilute acid solutions, which greatly limits its applications. In order to improve its solubility and widen the applications, increasing attention has been paid to the modification of chitosan by acylation reactions, graft copolymerization, physical and enzymatic methods [7,8]. Among these modification methods, graft copolymerization has been used most extensively [9].

Recently, many phenolic antixodants including gallic, caffeic and ferulic acids have been grafted onto chitosan in order to improve the water solubility as well as antioxidant activity of chitosan [10–16]. Caffeic acid (CA) and ferulic acid (FA) are two antioxidant phenolic acids extractable from plants [17,18]. It has been reported that CA and FA can be grafted onto chitosan by two methods, including the laccase catalyzed polymerization and carbodiimide mediated coupling reaction [10–12,16]. However, these two strategies both have some limitations. Laccase catalyzes the oxidation of phenolic acids into *o*-quinones which further react with the primary amines of chitosan. The loss of the active phenol hydroxyl groups might suppress some functional properties, *e.g.*, antioxidant activity of phenolic acids [10,12]. On the other hand, the carbodiimide mediated coupling reaction usually requires a large amount of chemical cross-link reagents. These chemical reagents used are environmentally disadvantageous and may cause adverse impacts on the human body when chitosan derivatives are added into food and medicine [11,16]. Moreover, previous studies have been mainly focused on the antioxidant activity *in vitro* of chitosan derivatives, concerning reducing power,

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2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and 2,2-azinobis-(-3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity [10–12,16]. It should be noted that antioxidants protect human body from ROS damages in both *in vitro* and *in vivo* models. In endogenous defense systems, antioxidants may act by up regulating the activities of enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px). As a result, many biomarkers including antioxidant enzyme levels and lipid peroxidation contents have been usually applied to measure the level of oxidation *in vivo* [19,20]. However, little attention has been paid to *in vivo* antioxidant activities of chitosan derivatives till now.

D-galactose (D-Gal) is a reducing sugar which can be metabolized by D-galactokinase and galactose-1-phosphate uridyltransferase in animals. However, overdose of D-Gal beyond the capacities of those two enzymes will allow aldose reductase to catalyze the accumulated D-Gal into galactitol, which cannot be metabolized and will accumulate in the cell, leading to osmotic stress and generation of ROS [21]. Recently, D-gal injected mice has been utilized as a model for anti-aging pharmacology and brain aging studies. Evidence has shown that D-gal caused aging-related changes in behavior and neuro-chemistry, as well as the increase of ROS and the decrease of antioxidant enzyme activity [22,23].

The aim of this study was to obtain water soluble chitosan derivatives by an eco-friendly method and evaluate their antioxidant activity *in vitro* and *in vivo*. Firstly, CA and FA were grafted onto chitosan by a free radical mediated grafting method, using ascorbic acid (Vc) and hydrogen peroxide (H_2O_2) redox pair system under inert atmosphere. Then, the synthesized CA grafted chitosan (CA-g-chitosan) and FA grafted chitosan (FA-g-chitosan) were characterized by UV–vis, Fourier-transform infrared (FT-IR) and nuclear magnetic resonance (NMR) spectroscopy to confirm the conjugation. The thermal behavior and crystallographic structure of the chitosan derivatives were determined by thermogravimetric analysis (TGA) and X-ray diffraction (XRD), respectively. Finally, the antioxidant activity *in vitro* of the chitosan derivatives was determined by measuring superoxide radical, hydroxyl radical and H_2O_2 scavenging activity as well as lipid peroxidation inhibition effect. The potential antioxidant activity *in vivo* of the chitosan derivatives was investigated by using D-Gal induced aging mice model. This study provides novel structural and *in vivo* antioxidant data for CA and FA grafted chitosan.

2. Materials and methods

2.1. Materials and reagents

Chitosan (degree of deacetylation, 71%) was obtained from Sangon Biotechnology Co. Ltd. (Shanghai, China). Folin–Ciocalteu reagent, Vc, CA, FA, deuterium oxide (D_2O) and pyrogallol were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Commercial kits used for determination of the superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) activities, and the contents of malondialdehyde (MDA) and protein were purchased from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). All other reagents were of analytical grade.

2.2. Syntheses of CA-g-chitosan and FA-g-chitosan

The syntheses of CA-g-chitosan and FA-g-chitosan were performed by using Vc and H_2O_2 redox pair under inert atmosphere according to our previously reported method [24]. Briefly, 0.25 g of chitosan was dissolved in 50 ml of 2% acetic acid solution (v/v) in a 0.5 L three-necked round bottom flask. Then, 0.1 g of Vc and 0.8 g of phenolic acid (CA or FA) were added into the reactor. A slow stream (1 ml/min) of oxygen free nitrogen gas was passed through the reactor for 30 min with stirring. Afterwards, 2 ml of 5 M H_2O_2 solution was added to initiate the reaction. The reaction was carried out under a continuous flow of oxygen free nitrogen gas for 12 h. The reaction mixture was dialyzed against distilled water with a 14,000 Da molecular weight cutoff membrane for 72 h to remove unreacted phenolic acids. Finally, the dialyzate was lyophilized to afford water-soluble CA-g-chitosan and FA-g-chitosan samples, respectively.

2.3. Determination of grafting ratios

The grafting ratios of CA-g-chitosan and FA-g-chitosan were measured by the Folin–Ciocalteu method with slight modifications [25]. Briefly, an aliquot of 1 ml of sample solution was mixed with 1 ml of Folin–Ciocalteu reagent and allowed to react at 30 °C for 5 min in the dark. Then, 5 ml of saturated Na_2CO_3 solution was added and the mixture was allowed to stand for 2 h before the absorbance of the reaction mixture was read at 760 nm. CA and FA were used to calculate the standard curves, respectively. The grafting ratios of CA-g-chitosan and FA-g-chitosan were expressed as mg of CA equivalents per g (mg CAE/g) and mg of FA equivalents per g (mg FAE/g), respectively.

2.4. Characterization of CA-g-chitosan and FA-g-chitosan

The structures of CA-g-chitosan and FA-g-chitosan were characterized by UV–vis, FT-IR and NMR spectra. The UV–vis spectra of CA-g-chitosan and FA-g-chitosan were determined by a Lambda 35 spectrophotometer (PerkinElmer Ltd., USA). FT-IR spectra were recorded on a continuous scan Varian 670 FT-IR spectrometer (Varian Inc., USA) in the frequency range of 4000–400 cm^{-1}. Proton nuclear magnetic resonance (^1H NMR) spectra were recorded at 25 °C for samples dissolved in CD_3COOD/D_2O (1%, v/v) using an AVANCE-600 spectrometer (Bruker Inc., Germany). Solid-state ^{13}C NMR experiments were performed on an AVIII 400 MHz WB spectrometer (Bruker Inc., Germany) operating at a ^{13}C frequency of 100.62 MHz and equipped with a double resonance H/X CP-MAS 4 mm probe.

The thermal behavior and crystallographic structures of the chitosan derivatives were determined by TGA and XRD, respectively. TGA were performed by a PerkinElmer Pyris 1 TGA (PerkinElmer Ltd., USA) heating from 30 to 800 °C at a rate of 10 °C/min under nitrogen atmosphere. Powder XRD spectra were acquired in the angular range of 10–80° (2θ) on a Bruker AXS D8 Advance X-ray diffractometer (Bruker Inc., Germany) using Ni-filtered Cu Kα radiation.

2.5. Assay of antioxidant activity in vitro

2.5.1. Assay of superoxide radical scavenging activity

The superoxide radical scavenging activity was performed according to the method of Jing and Zhao with some modifications [26]. Reaction was carried out in a mixture containing 4.5 ml of 50 mM Tris–HCl buffer (pH 8.2), 0.4 ml of 25 mM pyrogallol solution and 1 ml of sample (0.05–1 mg/ml of CA-g-chitosan or FA-g-chitosan in Tris–HCl buffer) by incubating at 25 °C for 5 min. Then, 1 ml of 8 mM HCl solution was dripped into the mixture promptly to terminate the reaction. The absorbance of the mixture was measured at 420 nm. V_C was used as the positive control. The superoxide radical scavenging activity was calculated by the following formula:

$$\text{Scavenging activity}\,(\%) = \left[\frac{1-(A_1-A_2)}{A_0}\right] \times 100 \quad (1)$$